Supporting Information

Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA processing system

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Plasmid vector construction

To construct pRGE32, the rice *UBIp* fragment was amplified from genomic DNA of Nipponbare cultivar with a pair of specific primers (UBI-F and UBI-R, see Table S2 for primer sequences). After amplifying the *U3p-gRNA* fragment from pRGE31 (Addgene plasmid 50929) with primers UGW-U3-F and UGW-gRNA-R, the *U3p-gRNA* and *UBIp* fragments were linked together by overlapping extension PCR with primers UGW-U3-F and UBI-R. Then pRGE32 was constructed by Gibson Cloning (New England Biolabs) to replace the *U3p-gRNA-35S* fragment in pRGE31 with *U3p-gRNA-UBIp*. The pRGEB32 binary vector (see Fig. S2 for vector map) used for the *Agrobacterium*-mediated rice transformation was created by inserting the *U3p-gRNA-UBIp-Cas9* fragment from pRGE32 into pCAMBIA1301-BsaI with Gibson Cloning (New England Biolabs). The pCAMBIA1300-BsaI is derived from pCAMBIA1300 after removing all of BsaI sites through site-directed mutagenesis. The primers used in plasmid construction are listed in Table S2.

The pGTR plasmid, which contains a gRNA-tRNA fused fragment, was used as a template to synthesize PTGs in this study. To construct pGTR, the gRNA scaffold fragment was amplified by PCR using a pair of specific primers (Bsa-gRNA-F and gRNA-R) whereas the $tRNA^{Gly}$ fragment was amplified as a primer dimer of g-tRNA-F and tRNA-R. Then these two fragments were fused as gRNA-tRNA by overlapping extension PCR using primers Bsa-gRNA-F and tRNA-R. The overlapping PCR product was separated and purified from an agarose gel, and then inserted into pGEM-T easy (Promega) to generate pGTR plasmid. The sequence of gRNA-tRNA fusion in pGTR is shown in Table S1. The primers used in plasmid construction are listed in Table S2.

RNA extraction, cRT-PCR and quantitative PCR

Total RNAs were extracted from protoplasts using the TRIzol Reagent (Life Technologies) according to the manufacturer's instruction. The cRT-PCR was schematically depicted in Fig. S3 and performed as follow. To circularize RNA, 1 μg of

total RNA was incubated in a 20 µl reaction containing 1 × T4 RNA ligase buffer (New England Biolabs), 50 µM of ATP, 10% PEG8000, 20 U of RNase inhibitor (New England Biolabs) and 10 U of T4 RNA ligase (New England Biolabs). The ligation was carried out at 25 ^{o}C for 4 h. Then 10 U of DNase I (New England Biolabs) was added into ligation reaction to remove genomic DNA contamination at 25 ^{o}C for 20 min. Then circularized RNA was purified with TRIzol Reagent (Life Technologies) and dissolved in nuclease free water. A total of 200 ng circularized RNA was mixed with 0.5 mM dNTP and 1 µM of oligos specific to gRNA spacer (gRNA1-R and gRNA2-R, see Table S2) and denatured at 70 ^{o}C for 5 min. After chilling on ice, 1× MuMLV Reverse Transcriptase buffer, 20 U of RNase inhibitor (New England Biolabs), and 10 U of MuMLV reverse transcriptase (New England Biolabs) were added to synthesize 1st cDNA at 42 ^{o}C for 1 hour. The negative controls without adding MuMLV reverse transcriptase (-RT) were also performed for all samples. After reverse transcription, MuMLV was inactivated at 70 ^{o}C for 10 min. Then PCR was performed with the following 50 µl reaction: one twentieth of 1st cDNA, 1× Phusion HF buffer, 0.2 mM dNTPs, 0.5 µM of forward primers, 0.5 µM reverse primers, and 1 U of Phusion DNA polymerase (Thermo Scientific). The resulting PCR products were analyzed with 2% agarose gel electrophoresis. The cRT-PCR products were then sequenced after cloning into pGEM-T easy vector (Promega). See Table S2 for primer sequences used in cRT-PCR.

For quantitative RT-PCR, the DNase I treated total RNAs were reverse-transcribed to produce cDNAs using a specific primer gRNA-R along with MuMLV (New England Biolabs) according to the manufacturer's instructions. The real-time PCR was performed using GoTaq qPCR Master Mix (Promega) in the StepOnePlus Realtime PCR system (Life technologies). The gRNA1-F and gRNA-R primers were used for *sgRNA1* and *PTG1*, and gRNA2-F and gRNA-R primer were used for *sgRNA2* and *PTG2*. The rice *UBI* gene was used as the internal reference for relative quantification. See Table S2 for primer sequences.

To quantify deletion efficiency, quantitative PCR was performed using 10 ng of the

genomic DNA as a template to amplify the genomic fragments within *MPK5* or *UBI* loci. A pair of specific primers (MPK5-qF and MPK5-R611, Table S2) which encompass the gRNA2 cut site were used to amplify *MPK5* copies containing no fragment deletion (Fig. S1A). The relative quantity (RQ) of *MPK5* fragment without deletion was calculated using *UBI* as a reference gene. Because only *MPK5* fragments without deletion were amplified, the fragment deletion efficiency could be estimated as 100%-RQ (Table S4).

Synthesis of Polycistronic tRNA-gRNA (*PTG*) Genes by Golden Gate Assembly

The PTG genes were synthesized based on the principle of Golden Gate (GG) assembly which is broadly used to assemble DNA parts like customized transcription activator-like effector (TALE). Our assembly approach allows synthesizing PTGs with different combinations of gRNAs using the same components. For example, we made PTG3-PTG9 with the same set of oligo primers (Table S3). By hierarchical GG assembly reaction, two PTGs could be assembled together to create a longer PTG (like *PTG9*). PTGs with no more than 6 gRNAs (e.g. *PTG1-PTG8*) could be synthesized by one step GG assembly (Level 1, Fig. S6), whereas PTGs with more than 6 gRNAs (*e.g.*, *PTG9*) require two or more steps of GG assembly (Level 2, Fig. S7). The schematic diagrams of PTG synthesis approach are shown in Fig. S6 and S7, and details of primer design, GG assembly and plasmid construction are described as follow.

Step 1. Design primers to amplify gRNA-tRNA parts

In order to ligate multiple DNA parts in a desired order, GG assembly requires distinct 4-bp overhangs to ligate two DNA parts after digestion with BsaI (or other type II endonucleases such as AarI, BbsI, BsmAI, BsmBI). The gRNA spacer is the only unique sequence in *PTG* (Fig. 1*C*), thus PTGs should be divided into DNA parts within the gRNA spacer region. As shown in Fig. S6A and 6B, a gRNA spacer was split into two parts with 4 bp overlap and each half of the spacer was synthesized within oligo primers with a BsaI site. Details to design gRNA specific primers are described below:

1.1. Select a 4-bp long sequence within each gRNA spacer as BsaI overhangs in GG assembly. The overhang could be any 4 consecutive nucleotides of the gRNA spacer. Of note, DNA parts that assembled in the same GG reaction should have a distinct 4-bp overhang, and could not be 5'-*GGCA*-3' or 5'-*AAAC*-3' which are used in terminal adaptors for cloning to pRGE32 and pRGEB32 (Fig. S2).

1.2. Design the primer sequences as follow (also see Fig. S6):

In this example, the 9th to 12th nucleotides of a 20 nt long spacer of gRNA[*x*] is chosen as the BsaI overhang for GG cloning:

5'-N_1- N_2- N_3- N_4- N_5- N_6- N_7- N_8-N_9-N_{10}-N_{11}- N_{12}- N_{13}- N_{14}- N_{15}- N_{16}- N_{17}- N_{18}-N_{19}-N_{20}-3'

The primer should be:

gRNA[*x*]-F (Forward primer, anneal to 5'-end of gRNA scaffold):

5'-ta-*GGTCTC*-N-$N_9N_{10}N_{11}N_{12}$$N_{13}N_{14}N_{15}N_{16}N_{17}N_{18}N_{19}N_{20}$-gttttagagctagaa-3'

gRNA[*x*]-R (Reverse primer, anneal to 3'-end of pre-tRNA):

5'-cg-*GGTCTC*-N- $N_{12}N_{11}$ N_{10} N_9 N_8 N_7 N_6 $N_5N_4N_3N_2$ N_1- tgcaccagccggg-3'

Note: Any 4 consecutive nucleotides in the spacer could be selected as overhangs for GG assembly. This allows the selection of a specific overhang for each DNA part in GG assembly. The two 5'-terminal bases (shown in lowercase) are randomly added nucleotides to enhance BsaI digestion of PCR products. The red color indicates the reverse complementary sequence to the spacer. The lowercase letters at the 3'-end indicate bases that anneal to gRNA (Forward primer) or tRNA (Revers primer).

Step 2. Level 1 GG assembly (Construction of *PTG1-PTG8* genes)

See Fig. S6*C* for the overall strategy of level 1 GG assembly of PTGs.

2.1. Set up 50 µL PCR reactions to amplify DNA parts for PTG construction.

pGTR plasmid	0.1 ng
5X Phusion HF buffer	10 µl
dNTPs (10mM)	1 µl
Forward primer (10 µM)	2.5 µl
Reverse Primer (10 µM)	2.5 µl
Phusion (2U/µl, NEB)	0.5 µl
H_2O	*x* µl
Total	50 µl

To construct PTGs used in this study, the forward and reverse primers to amplify level 1 parts were added as follow:

PCR ID	Forward primer	Reverse primer	Level 1 parts symbol
P1	L5AD5-F	gR1-R	L5AD-gR1
P2	gR1-F	L3AD5-R	gR1-L3AD
P3	L5AD5-F	gR2-R	L5AD-gR2
P4	gR2-F	L3AD5-R	gR2-L3AD
P5	gR1-F	gR2-R	gR1-gR2
P6	L5AD5-F	gR3-R	L5AD-gR3
P7	gR3-F	gR4-R	gR3-gR4
P8	gR4-F	L3AD5-R	gR4-L3AD
P9	L5AD5-F	gR5-R	L5AD-gR5

P10	gR5-F	gR6-R	gR5-gR6
P11	gR6-F	L3AD5-R	gR6-L3AD
P12	L5AD5-F	gR7-R	L5AD-gR7
P13	gR7-F	gR8-R	gR7-gR8
P14	gR8-F	L3AD5-R	gR8-L3AD
P15	gR2-F	gR3-R	gR2-gR3
P16	gR8-F	gR5-R	gR8-gR5
P17	gR4-F	gR7-R	gR4-gR7

PCRs were run with the following program:

Temperature	Time	Cycles
98 °C	2 min	1
98 °C	10 sec	35
50 °C	20 sec	
72 °C	20 sec	
72 °C	2.5 min	1
4 °C	Hold	1

2.2. The PCR products were purified with Spin Column PCR Products Purification kit (BioBasic).

2.3. Individual parts were ligated together by GG assembly with the following reaction:

Level 1 parts	25-50 ng (add equal amount for each parts)
2 x T7 DNA ligase Buffer (NEB)	10 µl
Bovine Serum Albumin (1mg/ml)	2 µl
Bsa I (10 U/µl, NEB)	0.5 µl
T7 DNA Ligase (3000 U/µl, NEB)	0.5 µl
Total Volume	20 µl

For GG assembly reactions to construct *PTG1-PTG8*, the level 1 parts were added as follow:

Gene ID	**Encoding gRNAs**	**Level 1 parts used**
PTG1	gRNA1	L5AD-gR1 and gR1-L3AD
PTG2	gRNA2	L5AD-gR2 and gR2-L3AD
PTG3	gRNA3-gRNA4	L5AD-gR3, gR3-R4, and gR4-L3AD
PTG4	gRNA5-gRNA6	L5AD-gR5, gR5-gR6, and gR6-L3AD
PTG5	gRNA7-gRNA8	L5AD-gR7, gR7-gR8, and gR8-L3AD
PTG6	gRNA1-gRNA2	L5AD-gR1, gR1-gR2, and gR2-L3AD
PTG7	gRNA1-gRNA2-gRNA3-gRNA4	L5AD-gR1, gR1-gR2, gR2-gR3, gR3-gR4, and gR4-L3AD
PTG8	gRNA7-gRNA8-gRNA5-gRNA6	L5AD-gR7, gR7-gR8, gR8-gR5, gR5-gR6, and gR6-L3AD

2.4. GG reactions were performed in a thermal cycler (Bio-Rad) by incubation at 37 °C, 5 min and 20 °C, 10 min for 30-50 cycles; and then held at 20 °C for 1 hour.

2.5. The GG reaction product was diluted with 180 µl of H2O.

2.6. The level 1 GG assembly products were amplified in the 50 µl PCR reaction:

Ligation product (1:10 dilution)	1 µl
5 X Go Green buffer (Promega)	10 µl
dNTPs (10mM)	1 µl
S5AD5-F (10 µM)	1 µl
S3AD5-R (10 µM)	1 µl
GoTaq DNA polymerase (2U/µl, Promega)	1 µl
H2O	35 µl
Total	50 µl

PCR was run in a thermal cycler (Bio-Rad) with the following program:

Temperature	Time	Cycles
95 °C	2 min	1
95 °C	10 sec	35
60 °C	20 sec	
72 °C	1 min/kb	
72 °C	5 min	1
4 °C	Hold	1

2.7. Purify the PCR product with Spin Column PCR Products Purification kit (Bio Basic).

2.8. Digest the purified PCR product with Fok I (NEB).

2.9. Separate the Fok I digested products in 1% agarose gel; Excise the DNA bands with the expected size from the gel and then purify them with Spin Column DNA Gel Extraction Kit (Bio Basic).

2.10. Ligate the Fok I digested GG fragment into the BsaI digested pRGE32 or pRGEB32 vectors with T4 DNA ligase (NEB).

2.11. Transform the ligation product to *E. coli* DH5α, purify the recombinant plasmids and confirm the constructs by Sanger sequencing.

Step 3. Level 2 GG assembly (Construct *PTG9* by two-step GG assembly)

The schematic diagram of level 2 GG assembly is shown in Fig.S7. Level 2 GG assembly is used to synthesize PTGs with more than six gRNAs. Such a large PTG is constructed by ligating two smaller PTGs (level 2 parts) together. These two small PTGs are synthesized by level 1 GG assembly and contain one overlapped gRNA as a bridge (bridge gRNA, Fig. S7) to ligate level 2 parts in a next GG assembly reaction. To amplify level 2 parts from level 1 assembled PTGs, a pair of specific primers annealing only to the bridge gRNA spacer are required for PCR with the terminal adaptor primers (S5AD5-F and S3AD5-R). In this study, the *PTG9* was synthesized with this approach and gRNA7 was used as the bridge gRNA.

3.1. Design bridge gRNA spacer-specific primers to amplify level 2 parts.

Bridge gRNA spacer:

5'-N_1- N_2- N_3- N_4- N_5- N_6- N_7- N_8-N_9-N_{10}-N_{11}- N_{12}- N_{13}- N_{14}- N_{15}- N_{16}- N_{17}- N_{18}-N_{19}-N_{20}-3'

Any consecutive 4-bp could be selected as an overhang for GG assembly. In this example, N_9-N_{10}-N_{11}-N_{12} was selected as an overhang.

Ln-gR[*x*]-F (Forward primer, only annealing to bridge gRNA spacer):

5'-ta-*GGTCTC*-N-$N_9N_{10}N_{11}N_{12}N_{13}N_{14}N_{15}N_{16}N_{17}N_{18}N_{19}N_{20}$-3'

Ln-gR[*x*]-R (Reverse primer, only annealing to bridge gRNA spacer):

5'-cg-*GGTCTC*-N- $N_{12}N_{11}$ N_{10} N_9 N_8 N_7 N_6 $N_5N_4N_3N_2$ N_1-3'

Note: The two 5'-terminal bases (shown in lowercase) are randomly added nucleotides to enhance Bsa I digestion of PCR products. The red color indicates the reverse complementary sequence to the spacer.

3.2. Set up GG reactions with the following level 1 parts generated from 2.1-2.2.

PTG ID	Encoding gRNAs	Level 1 Parts used in GG assembly reaction
PTG7-Ln	gRNA1-gRNA2-gRNA3-gRNA4-gRNA7	L5AD-gR1, gR1-gR2, gR2-gR3, gR3-R4, gR4-gR7, and gR7-L3AD

The level 1 GG assembly was carried out in the same manner as steps 2.4-2.5.

3.3. Amplify level 2 DNA parts with the following primers and templates:

PCR ID	Forward primer	Reverse primer	Template (1:10 diluted Level 1 GG reaction)
L2-P1	S5AD5-F	Ln-gR7-R	PTG7-Ln (step 3.2)
L2-P2	Ln-gR7-F	S3AD5-R	PTG8 (step 2.5)

The PCR condition is the same as step 2.6.

3.4. Separate the PCR products in 1% agarose gel. Excise the PCR bands with the expected size from the gel and purify them with Spin Column DNA Gel Extraction kit (Bio Basic).

3.5. Set up GG assembly reactions to ligate two DNA parts (L2-P1 and L2-P2) together.

L2-P1 PCR product	50 ng
L2-P2 PCR product	50 ng
2 x T7 DNA ligase Buffer (NEB)	10 µl
Bovine Serum Albumin (1mg/ml)	2 µl
Bsa I (10 U/µl, NEB)	0.5 µl
T7 DNA Ligase (3000 U/µl, NEB)	0.5 µl
Total Volume	20 µl

3.6 Perform GG assembly reactions in a thermal cycler (Bio-Rad) using the following program: 37 ^{o}C, 5 min; 20 ^{o}C, 10 min for 25 cycles; and 20 ^{o}C for 1 hour.

3.7. Amplify Level 2 GG assembled product with S5AD5-F and S3AD5-R, then inserted the amplified product into pRGE32 or pRGEB32 using the same procedure as steps 2.6-2.11.

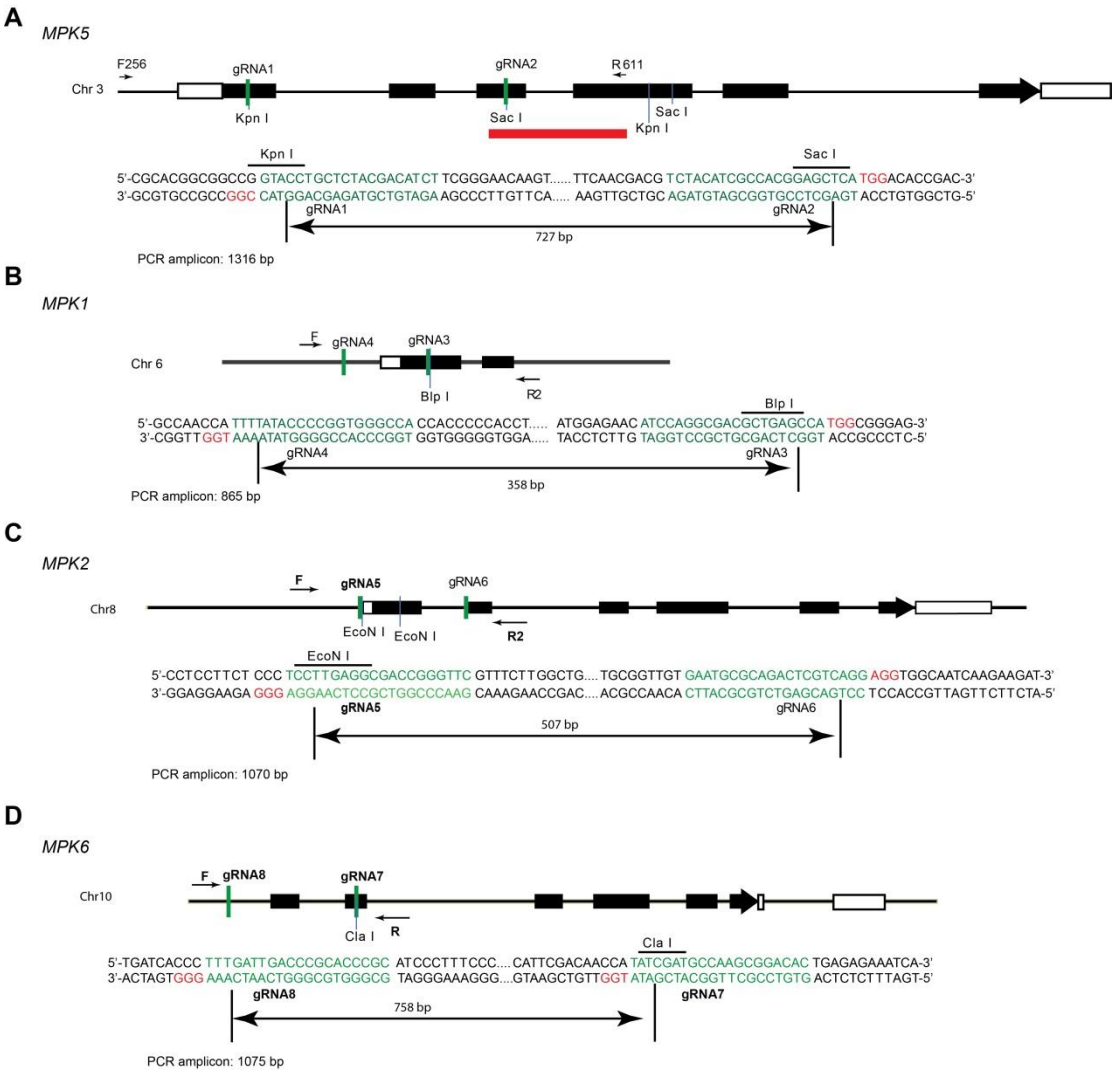

Fig. S1. Schematic diagram of rice MAPK genes targeted by gRNA1-gRNA8. The rectangles indicate exons while black rectangles indicate protein coding region. The relative location of gRNA targeting site is shown as green vertical line and PCR primers for genotyping are indicated with black arrow. The gRNA targeting regions (green letters), PAMs (red letters) and restriction enzyme sites for PCR/RE assay are also shown for each gene. The red bar in A indicates the qPCR amplicon for deletion efficiency estimation (Table S4). Only a partial region of *MPK1* locus is shown here.

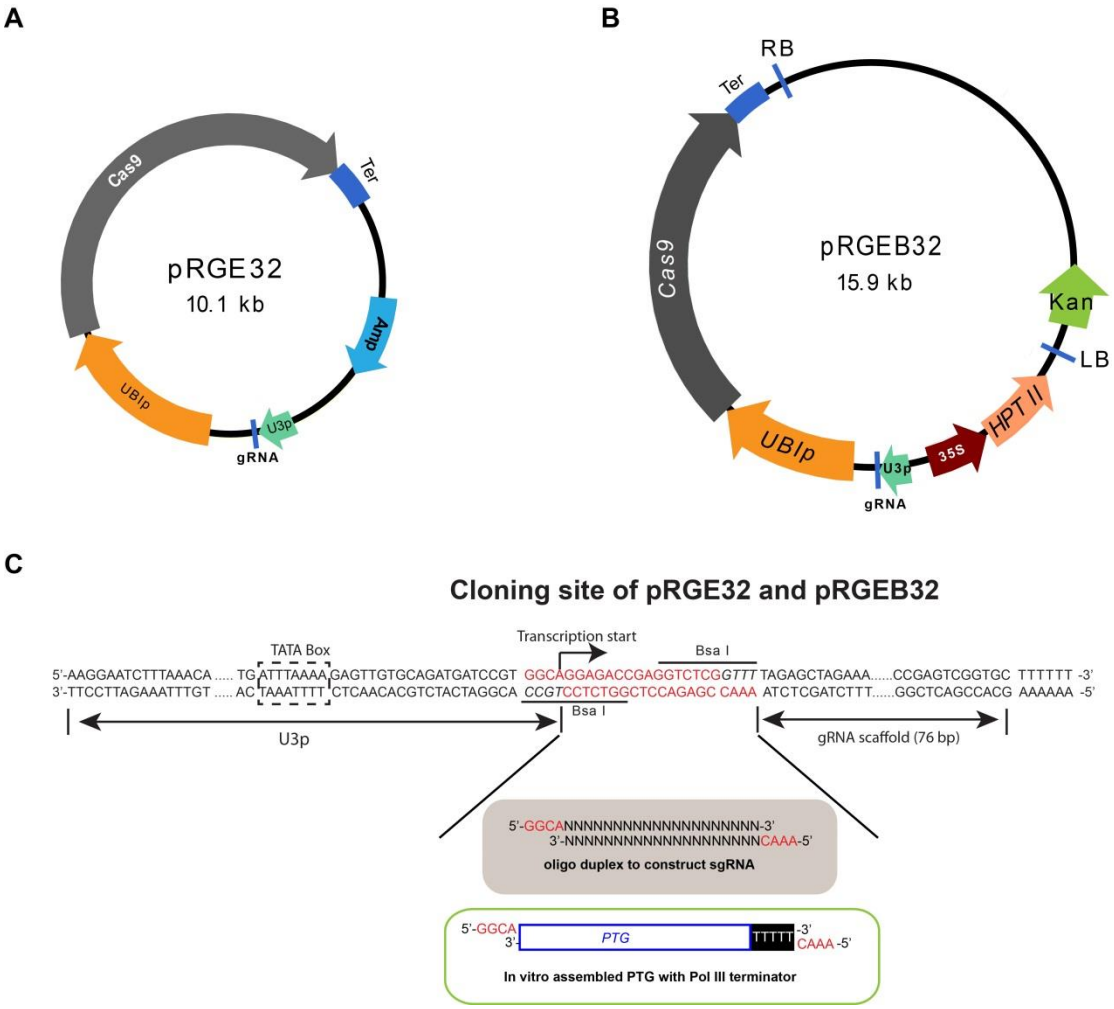

Fig. S2. Illustration of pRGE32 and pRGEB32 plasmids used in this study. (**A** and **B**) Schematic depiction of pRGE32 and pRGEB32 plasmid vectors. The pRGE32 vector was used for transient expression of sgRNAs and PTGs along with Cas9 in plant protoplasts, and pRGEB32 is a binary vector for the *Agrobacterium*-mediated transformation. (**C**) The cloning site for insertion of gRNA spacer sequence or *PTG* genes into both vectors. The red letters in vector indicate the cut off fragment and italic letters indicates overhangs in linearized vectors after BsaI digestion. The appropriate overhangs of oligo-duplex or synthetic PTG genes are shown at the bottom. Amp, ampicillin resistance gene; Kan, kanamycin resistance gene; 35S, cauliflower mosaic virus 35S promoter; UBIp, rice ubiquitin promoter; U3p, rice U3 snoRNA promoter; HPT II, hygromycin phosphotransferase II; Ter, nopaline synthase terminator; LB, T-DNA left border; RB, T-DNA right border.

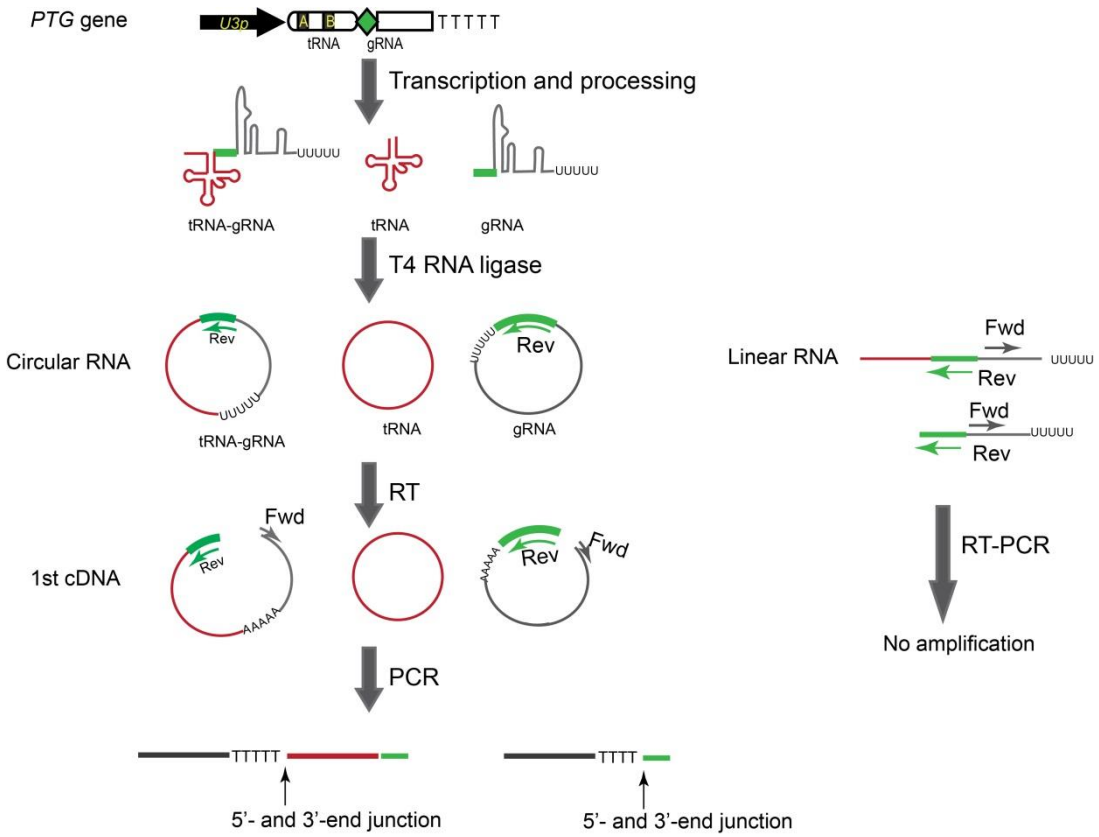

Fig. S3. Schematic depiction of cRT-PCR to map gRNA 5' and 3' ends. The primary *PTG* transcript or processed mature gRNA could be circularized by self-ligation with T4 RNA ligase. The circular RNA was reverse-transcribed to cDNA with gRNA spacer-specific primers (Rev). Then the fragment containing 5'- and 3'-end junction could be amplified with a pair of specific primers (Fwd and Rev) to obtain sequences with mature gRNA extremities. In the cRT-PCR, the linear RNA fraction could not be amplified (right panel) because the primers were not paired.

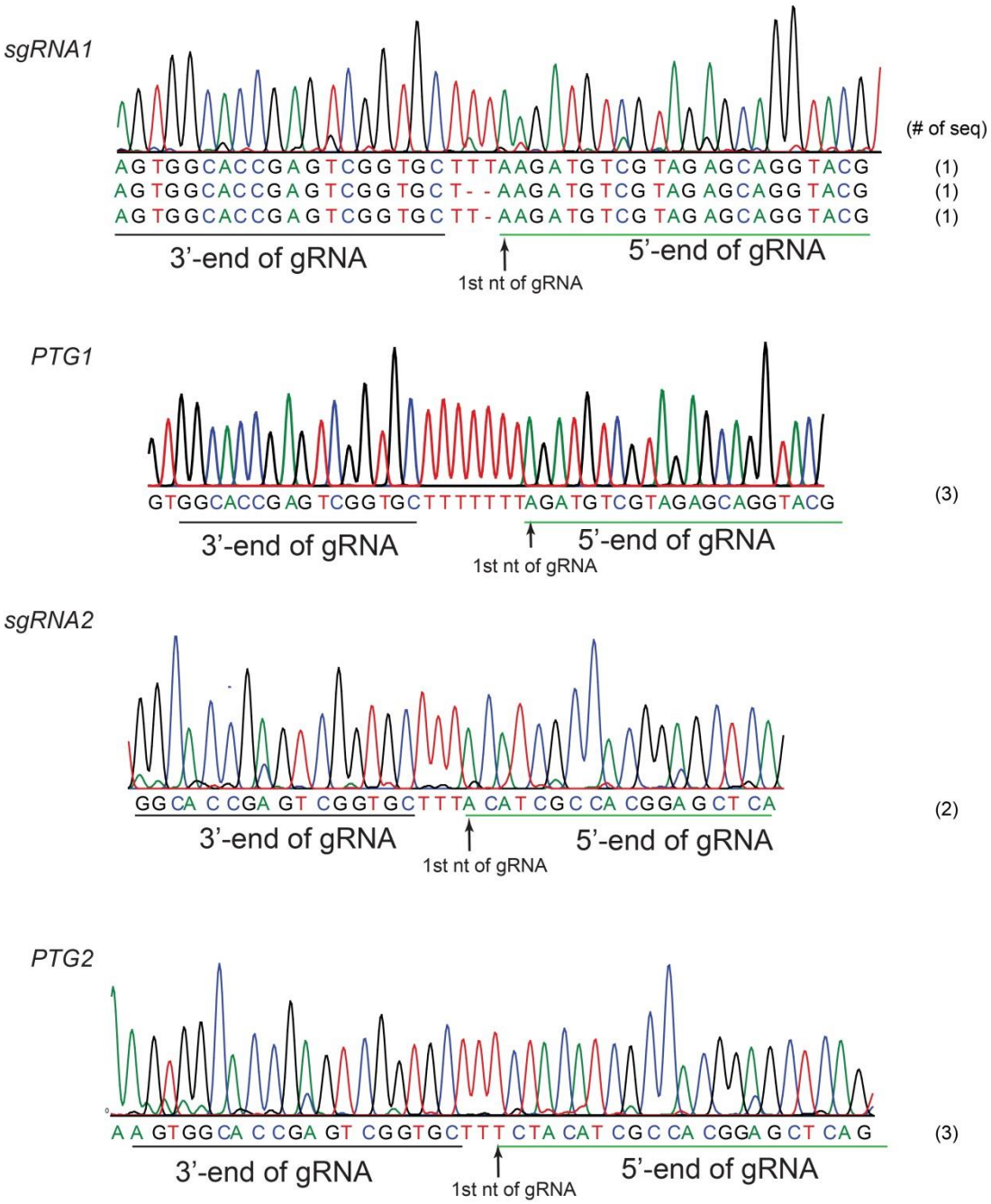

Fig. S4. Sequences of cRT-PCR products from *sgRNA1*, *sgRNA2*, *PTG1* and *PTG2*. An example of sequence chromatographs is shown for each cRT-PCR product. The number of individual colonies with the identical sequences is shown in parentheses. The sequencing results indicate that variable length of poly(U) tails were added to the 3'-end of gRNAs produced by these genes.

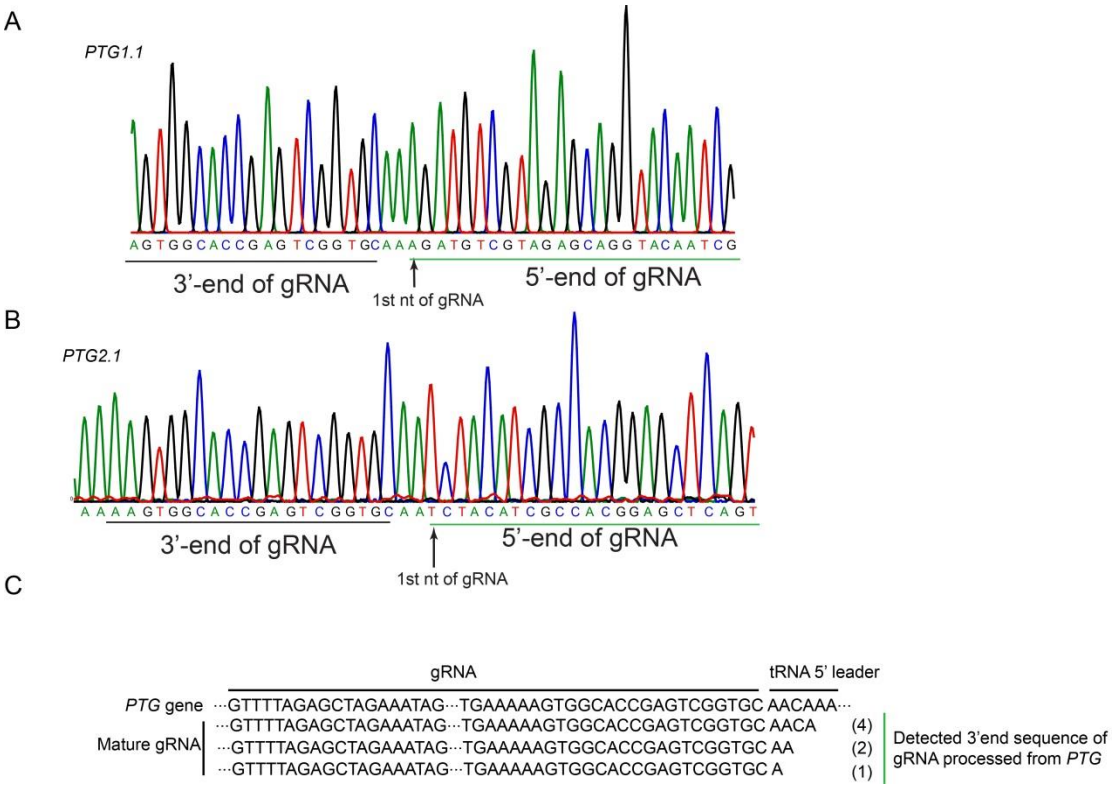

Fig. S5. Sequences of cRT-PCR products from *PTG1.1* and *PTG2.1*. (**A** and **B**) Representative sequencing chromatographs of cRT-PCR products. (**C**) Summary of the mapped 3'-end of mature gRNAs from *PTG1.1* and *PTG2.1*. All sequences show identical 1st nt of gRNA 5'-end. The number of individually cloned PCR products with the identical sequences is shown in parentheses. Dots indicate bases not shown in the alignment.

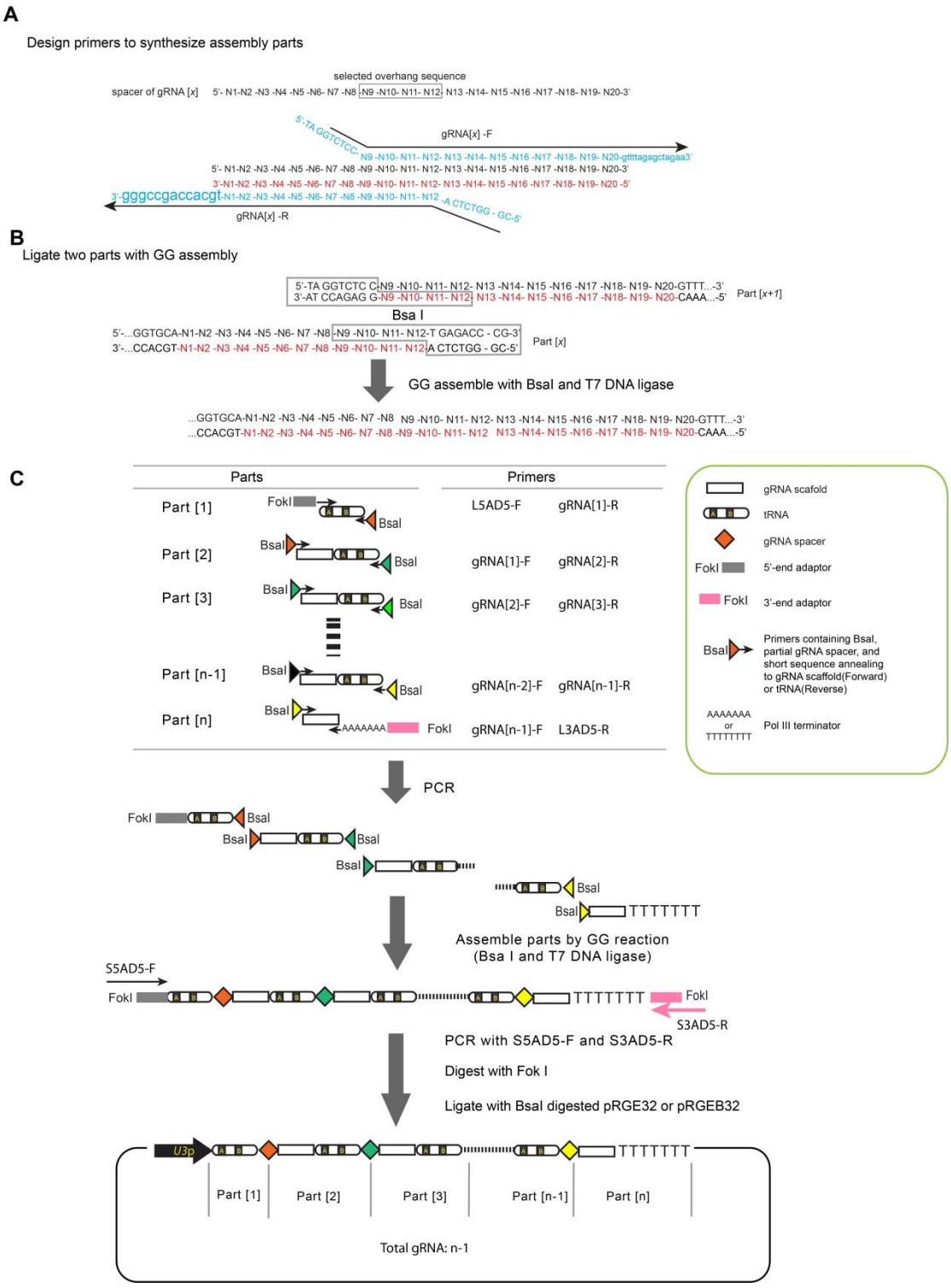

Fig. S6. Strategy to synthesize PTGs with one step (level 1) Golden Gate (GG) assembly. (**A)** Schematic guide to design gRNA spacer specific primers with 4 bp overlapping for Golden Gate assembly. The primers could be overlapped on any 4 consecutive nucleotides within the spacer. **(B**) Mechanism to generate a complete gRNA spacer during GG assembly. After PCR amplification and BsaI digestion, the 4 bp overlapped sequence in the gRNA spacer was generated as overhangs to ligate two parts and the resulting ligation product would produce a complete gRNA without

extra nucleotides. The DNA sequences in the box indicate BsaI cut site. (**C**) Schematic diagrams for level 1 GG assembly to synthesize PTGs from PCR parts and clone them into plasmid vectors (pRGE32 or pRGEB32). A PTG with *n-1* gRNAs are divided into *n* parts (Part[*1*] — Part[*n*], see the bottom). Each part was amplified with spacer-specific primers containing BsaI adaptor, except two terminal parts using gRNA spacer primer and terminal specific primers containing Fok I site (L5AD5-F and L3AD5-R). These PCR parts were ligated together using GG assembly to produce the PTG with complete gRNA spacers. The assembled product was amplified with short terminal specific primers (S5AD5-F and S3AD5-R). After Fok I digestion, the PTG fragment was inserted into the BsaI digested pRGE32 and pRGEB32. See Table S2 and Table S3 for primer sequences and SI Methods for details.

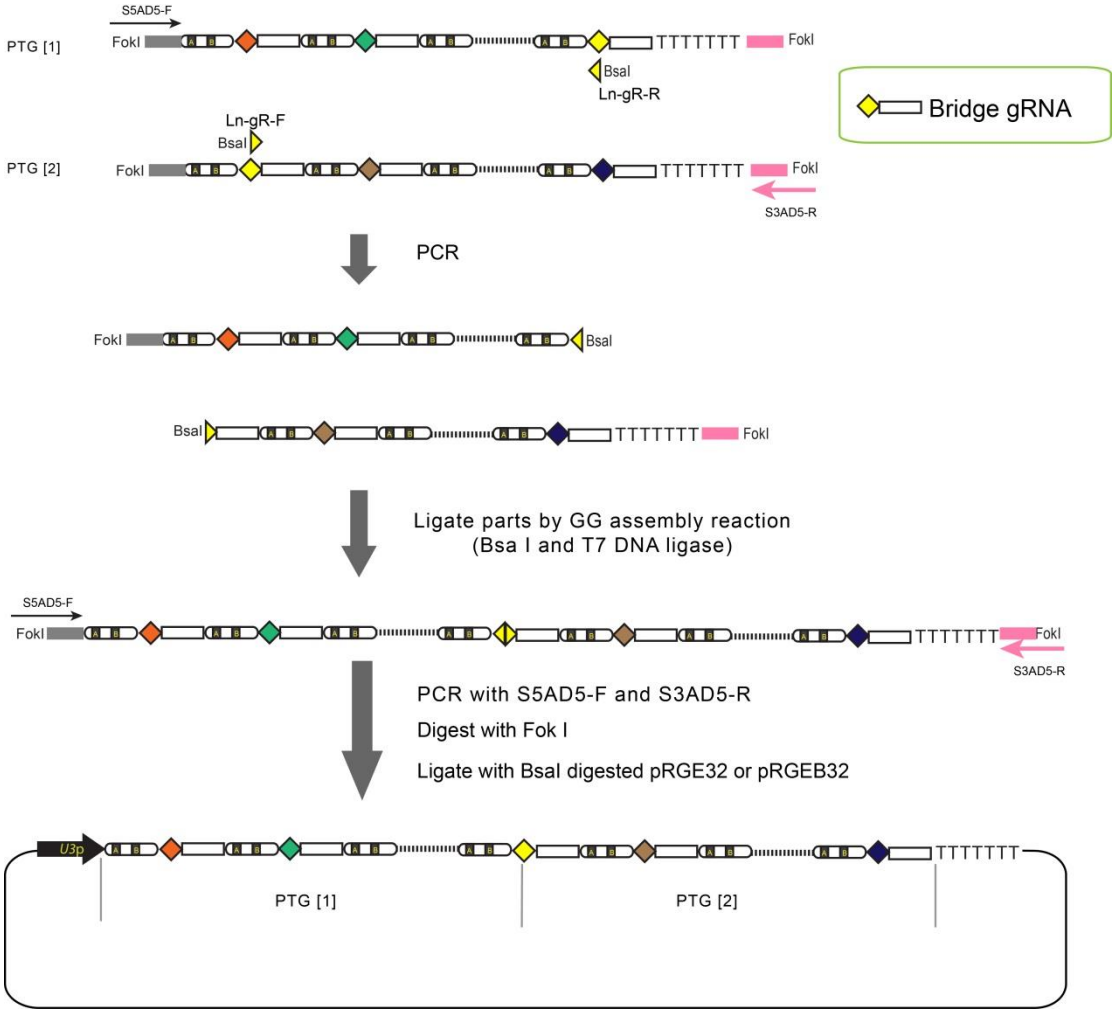

Fig. S7. Strategy to synthesize a large PTG from two PTG parts using level 2 Golden Gate (GG) assembly. Two PTG genes (PTG[1] and PTG[2]) containing different numbers (2–6) of gRNAs were synthesized by level 1 GG assembly (Fig. S6). To concatenate PTG[1] with PTG[2], the last gRNA (bridge gRNA) in PTG[1] should contain the same spacers (show in yellow diamond) as the first gRNA in PTG[2]. Then level 2 GG parts were amplified from PTG[1] and PTG[2] with bridge gRNA spacer specific primers (Ln-gR-F and Ln-gR-R) containing BsaI site and terminal specific primers (S5AD5-F and S3AD5-R). After Fok I digestion, these two PTG parts were ligated together with GG assembly and inserted into BsaI digested pRGE32 or pRGEB32 vector. See experimental details in SI Methods.

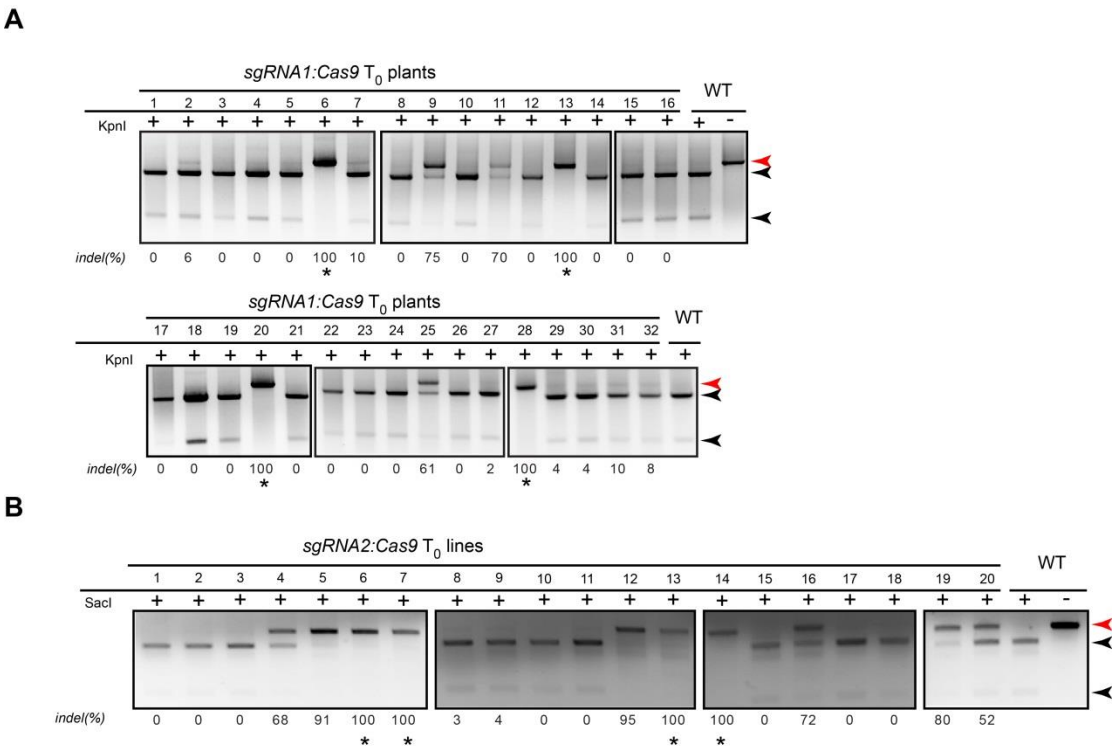

Fig. S8. Targeted mutagenesis at *MPK5* locus in transgenic plants expressing sgRNA:Cas9. Targeted DNA fragment was amplified from independent transgenic plants and analyzed with PCR/RE assays, and a wild type plant (WT) was included as control. Kpn I and SacI were used to examine mutation at sgRNA1/gRNA1 (**A**) and sgRNA2/gRNA2 (**B**) targets, respectively. On the agarose gels, mutated DNA fragments resistant to KpnI or SacI digestion are indicated with red arrow whereas digested WT DNA fragments are indicated with black arrow. The indel frequency, which was estimated based on the intensity of digested and undigested bands, is indicated at the bottom of each lane. The putative biallelic mutants are indicated by asterisk.

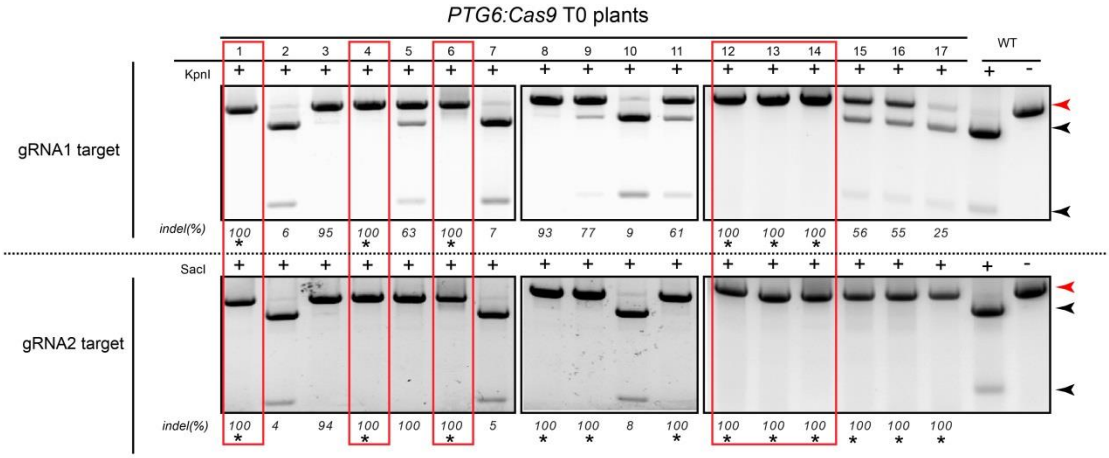

Fig. S9. Targeted mutagenesis at *MPK5* locus in transgenic plants expressing PTG6:Cas9. Mutations at *MPK5* locus of PTG6:Cas9 plants were examined with PCR/RE assay. The putative biallelic mutants at gRNA1 or gRNA2 site are indicated with asterisk, and biallelic mutations at both sites are indicated with red rectangle. In the gel, mutated DNA fragments resistant to restriction enzyme (KpnI or SacI) digestion are indicated with red arrow whereas digested WT DNA fragments are indicated with black arrow. The indel frequency, which was estimated based on the intensity of digested and undigested bands, is indicated at the bottom of each lane.

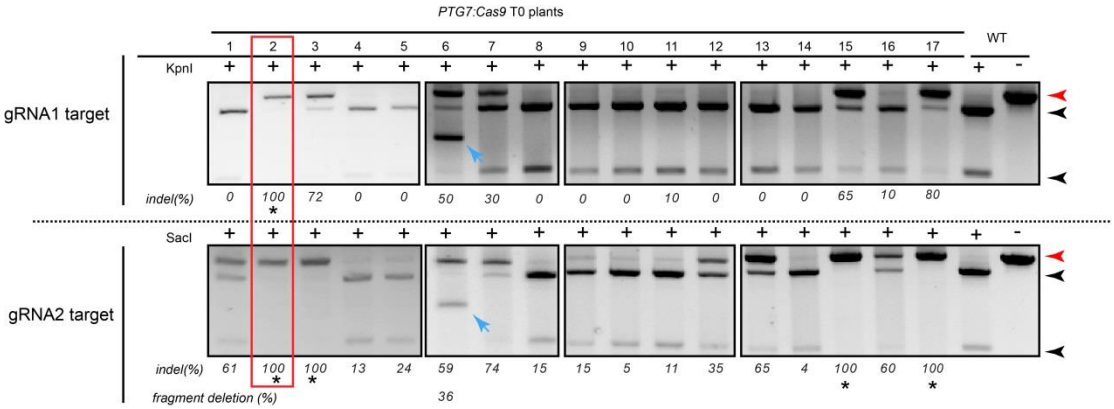

Fig. S10. Targeted mutagenesis at *MPK5* locus in transgenic plants expressing PTG7:Cas9. The mutations at gRNA1 and gRNA2 targets within MPK5 were examined by PCR/RE assays. The putative biallelic mutations at gRNA1 or gRNA2 site are indicated with asterisk, and biallelic mutations at both sites are marked with red rectangle. The deletion of a chromosomal fragment between gRNA1 and gRNA2 is shown with blue arrow. Mutated DNA fragments resistant to restriction enzyme digestion are indicated with red arrow whereas digested WT DNA fragments are indicated with black arrow. The indel frequency is indicated at the bottom of each lane.

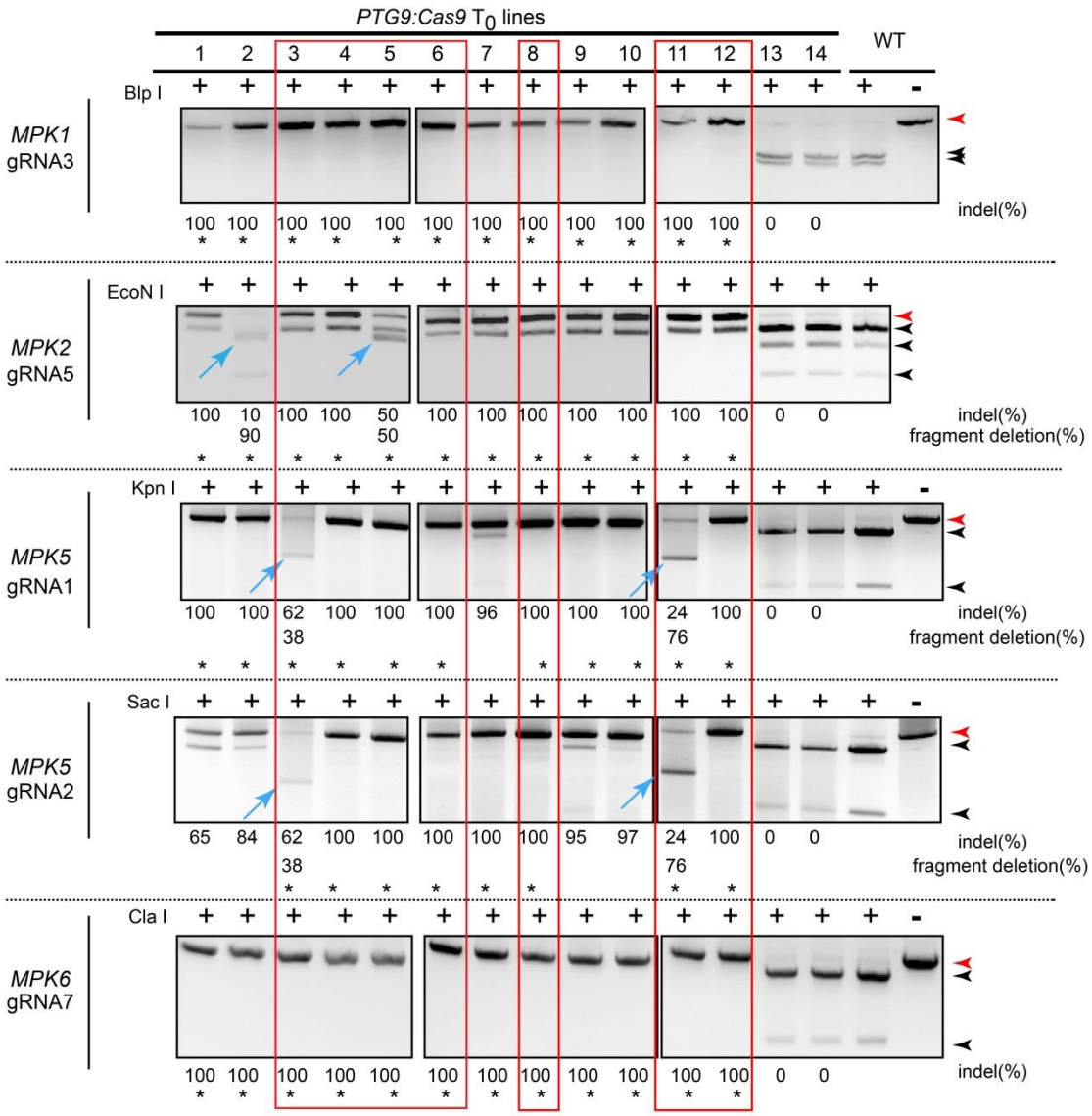

Fig. S11. Targeted mutagenesis at five genomic sites in transgenic plants expressing PTG9:Cas9. The mutations at the targeting sites of gRNA1/2/3/5/7 were examined by PCR/RE assays. The transgenic lines carrying potential biallelic mutations at each target are indicated with asterisk, and biallelic mutations at all five sites are marked with red rectangle. The deletion of chromosomal fragments between paired gRNAs is indicated with blue arrow. Mutated DNA fragments resistant to restriction enzyme digestion are indicated with red arrows whereas digested WT DNA fragments are indicated with black arrow. The indel frequency and fragment deletion frequency in each line are indicated at the bottom. Of note, there are two EcoN I sites within the *MPK2* amplicon and therefore the digestion of WT DNA fragment yielded three bands.

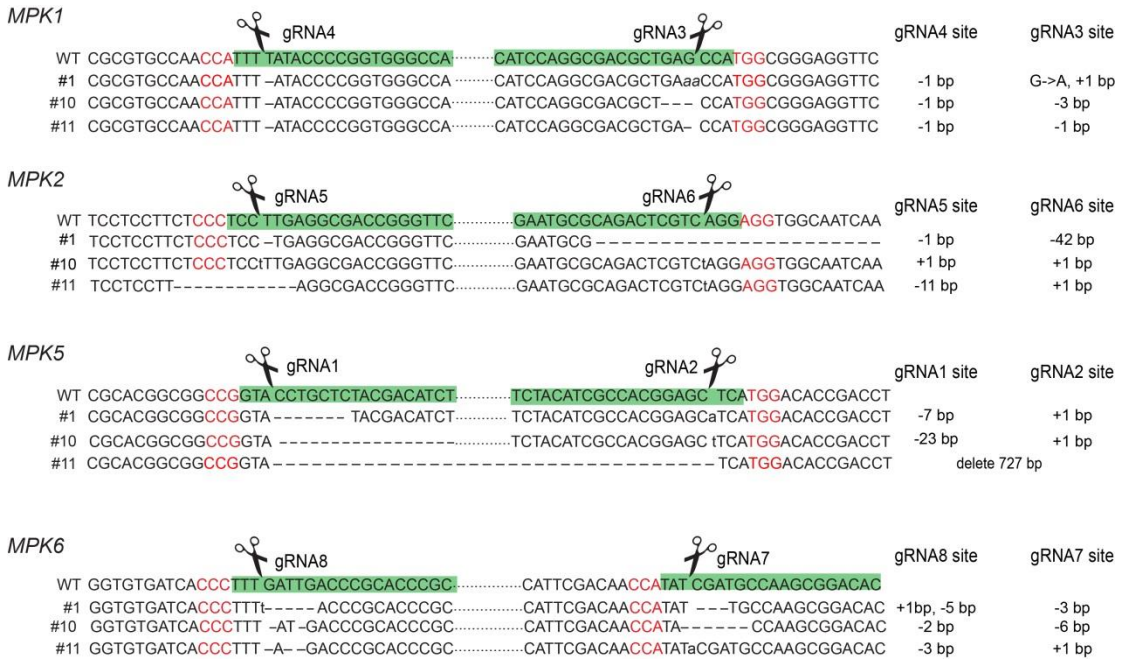

Fig. S12. Targeted mutation at eight genomic sites in four *MPK* loci in PTG9:Cas9 lines. The mutated sequences from three transgenic lines (#1, #10 and #11) expressing PTG9:Cas9 were aligned with wild type sequences. Insertion or substitution is shown in lowercase. Short line (-) indicates base pair deletion and dot (.) indicates sequences not shown in the alignments. The Cas9/gRNA cut sites are indicated with scissor.

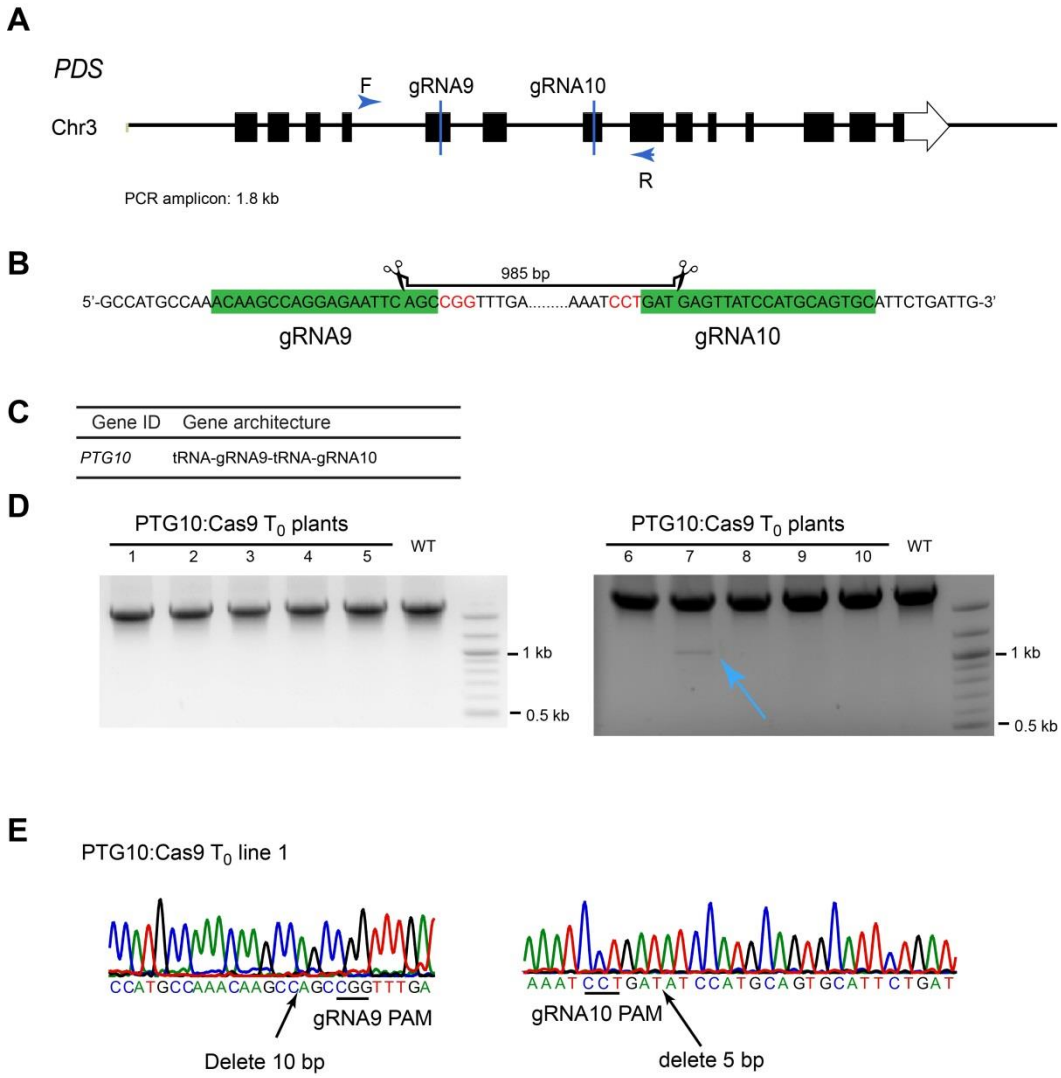

Fig. S13. Targeted mutation of rice *PDS* gene in transgenic plants with PTG10:Cas9. (A) Schematic depiction of rice *PDS* locus. Rectangles represent exons, of which the black ones indicate coding regions. The targeting sites of gRNA9 and gRNA10 and the location of primers (F and R) for genotyping are indicated with blue line and arrow, respectively. (**B**) Targeting sequences of gRNA9 and gRNA10 are highlighted in green color, and the relevant PAM sites are indicated with red letters. The predicted cut sites of Cas9:gRNA are indicated with scissor. (**C**) Gene architecture of *PTG10*. (**D**) PCR amplification of targeted DNA sites from transgenic plants expressing *PTG10:Cas9*. The deletion of chromosomal fragment between gRNA1 and gRNA2 is indicated with blue arrow. (**E**) Examples of mutation at the *PDS* target sites.

SI Tables

Table S1. Sequence of synthetic genes used in this study

Gene (Architecture)	**Sequence (5'->3')**
Sequence of gRNA-tRNA fusion in pGTR plasmid	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA
sgRNA1	GATCCGTGGCAAGATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT
sgRNA2	GATCCGTGGCAGTCTACATCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT
PTG1 (tRNA-gRNA1)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAGATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTT
PTG2 (tRNA-gRNA2)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATCTACATCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTT
PTG1.1 (tRNA-gRNA1-tRNA)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAGATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATTTTTTTTTT
PTG2.1 (tRNA-gRNA2-tRNA)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATCTACATCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATTTTTT

	TTTT
PTG3 (tRNA-gRNA3 -tRNA-gRNA4)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTA CCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAT CCAGGCGACGCTGAGCCAGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT CGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCT GCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATGGCC CACCGGGGTATAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT GCTTTTTTTTT
PTG4 (tRNA-gRNA5-t RNA-gRNA6)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTA CCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAG AACCCGGTCGCCTCAAGGAGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAG TCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCC TGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGAAT GCGCAGACTCGTCAGGGTTTTAGAGCTAGAAATAGCAAGTTAAA ATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG GTGCTTTTTTTTTT
PTG5 (tRNA-gRNA7-t RNA-gRNA8)	GATGATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATA GTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTG CAGTGTCCGCTTGGCATCGATAGTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGA GTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACC CTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGCG GGTGCGGGTCAATCAAAGTTTTAGAGCTAGAAATAGCAAGTTAA AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC GGTGCTTTTTTT
PTG6 (tRNA-gRNA1-t RNA-gRNA2)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTA CCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAA GATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT CGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCT GCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATCTACA TCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT GCTTTTTTTTTT
PTG7 (tRNA-gRNA1-t	GATCCGTGGCAAtAAAGCACCAGTGGTCTAGTGGTAGAATAGTAC CCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAG

RNA-gRNA2-tRNA-gRNA3-tRNA-gRNA4)	ATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATCTACATCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAATCCAGGCGACGCTGAGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATGGCCCACCGGGGTATAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTT
PTG8 (tRNA-gRNA7-tRNA-gRNA8-tRNA-gRNA5-tRNA-gRNA6)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGTGTCCGCTTGGCATCGATAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGCGGGTGCGGGTCAATCAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGAACCCGGTCGCCTCAAGGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGAATGCGCAGACTCGTCAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTT
PTG9 (tRNA-gRNA1-tRNA-gRNA2-tRNA-gRNA3-tRNA-gRNA4-tRNA-gRNA7-tRNA-gRNA8-tRNA-gRNA5-tRNA-gRNA6)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAGATGTCGTAGAGCAGGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATCTACATCGCCACGGAGCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCA

	CGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAATCCAGGCG ACGCTGAGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCA ACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGG TACAGACCCGGGTTCGATTCCCGGCTGGTGCATGGCCCACCGGG GTATAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTA GTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAA AGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACA GACCCGGGTTCGATTCCCGGCTGGTGCAGTGTCCGCTTGGCATCG ATAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCAC CAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCG GGTTCGATTCCCGGCTGGTGCAGCGGGTGCGGGTCAATCAAAGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC AACTTGAAAAAGCGGCACCGAGTCGGTGCAACAAAGCACCAGT GGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTT CGATTCCCGGCTGGTGCAGAACCCGGTCGCCTCAAGGAGTTTTA GAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACT TGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTC TAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGAT TCCCGGCTGGTGCAGAATGCGCAGACTCGTCAGGGTTTTAGAGC TAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA AAAGTGGCACCGAGTCGGTGCTTTTTTTTT
PTG10 (tRNA-gRNA9-t RNA-gRNA10)	GATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTGGAATAGTA CCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAA CAAGCCAGGAGAATTCAGCGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAG TCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCC TGCCACGGTACAGACCCGGGTCCGATTCCCGGCTGGTGCACACT GCATGGATAACTCATCGTTTTAGAGCTAGAAATAGCAAGTTAAAA TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG TGCTTTTTTTTTTT

The sequences are annotated as follows:
Last 10 bp of *U3p*: white background
gRNA scaffold: yellow background
pre-tRNA: green background
gRNA spacer: yellow background with red letters
Pol III Terminator (TTTT..T): grey background

Table S2. Primers used for plasmid construction, cRT-PCR, and genotyping

Oligo Name	Sequence (5'->3')	Purpose
gRNA1-R	AAACGTACCTGCTCTACGAC	cRT-PCR of gRNA1
gRNA2-R	AAACTGAGCTCCGTGGCGAT	cRT-PCR of gRNA2
Bsa-gRNA-F	GGAGACCGAGGTCTCGGTTTTAGAGCTAGAAATA	cRT-PCR and amplify gRNA
gRNA1-F	GGC AAGATGTCGTAGAGCAGGTAC	Quantitative RT-PCR
gRNA2-F	GTCTACATCGCCACGGAGCTCA	Quantitative RT-PCR
gRNA-R	GCACCGACTCGGTGCCAC	Quantitative RT-PCR
UBI-qF	TGGTCAGTAATCAGCCAGTTTG	Quantitative RT-PCR
UBI-qR	CAAATACTTGACGAACAGAGGC	Quantitative RT-PCR
MPK5-qF	GATCCCGCCGCCGATCCCTC	Quantitative PCR
g-tRNA-F	GCACCGAGTCGGTGC AACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTG	Construct pGTR. Overlapped region is underlined.
tRNA-R	CTGCCATGCACCAGCCGGGAATCGAACCCGGGTCTGTACCGTGGCAGGGTACTATTCTAC	
UBI-F	TGCATGCCTGCAGGTCCACAAATTCGGGTCAAGGCGG	Amplify *UBIp* to construct pRGE32 and pRGEB32
UBI-R:	CAAACTTGTTGATAACTATCTGCAAGAAATAATCACCAAAC	
UGW-U3-F	GACCATGATTACGCCAAGCTTAAGGAATCTTTAAACATACG	Construct pRGE32
UGW-gRNA-R	GGACCTGCAGGCATGCACGCGCTAAAAACGGACTAGC	
MPK1-F	GGGTCGGCACAGCATCTC	Genotyping of MPK1
MPK1-R2	TGCGCCTAAAAATCGAGGGT	
MPK2-F	TTTGGGAAGCATGTATGAAGC	Genotyping of MPK2
MPK2-R2	TATGCCAGCCAATGAGCCAA	
MPK5-F256	GCCACCTTCCTTCCTCATCCG	Genotyping of MPK5
MPK5-R611	GTTGCTCGGCTTCAGGTCGC	
MPK6-F	TTGACGCCCCAACATAAATAA	Genotyping of MPK6
MPK6-R	TGTTGCTGCCGCTTTTCT	
PDS-F	GGTAGAAATGCCATGCGGGA	Genotyping of PDS
PDS-R	ATTCAGCCGAACCTCACCAC	
L5AD5-F	CG GGTCTC A GGCA GGATG GGCAGTCTG GGCA ACAAAGCACCAGTGG	PTG synthesis and cloning A Fok I site (underlined) was used to generate compatible overhangs (labeled with red color) for cloning into pRGE32 and pRGEB32.
L3AD5-R	TA GGTCTC C AAAC GGATG AGCGACAGC AAAC AAAAAAAAAA GCACCGACTCG	
S5AD5-F	CG GGTCTC A GGCA GGATG GGCAGTCTG GGCA	
S3AD5-R	TA GGTCTC C AAAC GGATG AGCGACAGC AAAC	

Table S3. Oligo nucleotides used to synthesize *PTG* genes

gRNA ID (Target)	Spacer/Protospacer (5'->3') [a]	Oligo ID	Sequence (5'->3')[b]
gRNA1 (MPK5)	AGATGTCGTAGAGCAGGTAC	gR1-F	TA ***GGTCTCC*** TAGAGCAGGTAC gttttagagctagaa
		gR1-R	AT ***GGTCTCA*** TCTACGACATCT tgcaccagccgggaa
gRNA2 (MPK5)	TCTACATCGCCACGGAGCTCA	gR2-F	TA ***GGTCTCC*** CCACGGAGCTCA gttttagagctagaa
		gR2-R	AT ***GGTCTCA*** GTGGCGATGTAGA tgcaccagccgggaa
gRNA3 (MPK1)	ATCCAGGCGACGCTGAGCCA	gR3-F	TA ***GGTCTCC*** GACGCTGAGCCA gttttagagctagaa
		gR3-R	AT ***GGTCTCA*** CGTCGCCTGGAT tgcaccagccgggaa
gRNA4 (MPK1)	TGGCCCACCGGGGTATAAAA	gR4-F	TA ***GGTCTCC*** ACCGGGGTATAAAA gttttagagctagaa
		gR4-R	CG ***GGTCTCA*** CGGTGGGCCA tgcaccagccggg
gRNA5 (MPK2)	GAACCCGGTCGCCTCAAGGA	gR5-F	TA ***GGTCTCC*** GTCGCCTCAAGGA gttttagagctagaa
		gR5-R	CG ***GGTCTCA*** CGACCGGGTTC tgcaccagccggg
gRNA6 (MPK2)	GAATGCGCAGACTCGTCAGG	gR6-F	TA ***GGTCTCC*** CAGACTCGTCAGG gttttagagctagaa
		gR6-R	CG ***GGTCTCA*** TCTGCGCATTC tgcaccagccggg
gRNA7 (MPK6)	GTGTCCGCTTGGCATCGATA	gR7-F	TA ***GGTCTC C*** TTGGCATCGATA gttttagagctagaa
		gR7-R	AT ***GGTCTCA*** CCAAGCGGACAC tgcaccagccgggaa
gRNA8 (MPK6)	GCGGGTGCGGGTCAATCAAA	gR8-F	TA ***GGTCTCC*** CGGGTCAATCAAA gttttagagctagaa
		gR8-R	CG ***GGTCTCA*** CCCGCACCCGC tgcaccagccggg
gRNA7	Linkers for level 2 GG assembly	Ln-gR7-F	TA ***GGTCTC C*** TTGGCATCGATA
		Ln-gR7-R	AT ***GGTCTC A*** CCAAGCGGACAC
gRNA9 (PDS)	ACAAGCCAGGAGAATTCAGC	gR9-F	TA ***GGTCTC C*** CAGGAGAATTCAGC gttttagagctagaa
		gR9-R	CG ***GGTCTC A*** CCTGGCTTGT tgcaccagccggg
gRNA10 (PDS)	CACTGCATGGATAACTCATC	gR10-F	TA ***GGTCTC C*** ATGGATAACTCATC gttttagagctagaa
		gR10-R	CG ***GGTCTC A*** CCATGCAGTG tgcaccagccggg

[a] The boxed letters indicate the overhang sequences in Golden Gate assembly.

[b] The first two letters are randomly added nucleotides. Italic bold sequences indicate the BsaI sites (5'-*GGTCTCN-3'*, N indicates any nucleotide), underlined sequences are part of gRNA spacer whereas red-underlined sequences are overhangs after BsaI digestion. See SI Methods for details about primer design and PTG assembly. Sequences in lower case are specific for gRNA scaffold (5'-gttttagagctagaa-3', in forward primers) or tRNA (5'-tgcaccagccggg-3', in reverse primers).

Table S4. Determination of chromosomal fragment deletion frequency at the *MPK5* locus in rice protoplasts by qPCR

Sample	Gene	Ct mean	Ct SD	RQ	RQ-Min	RQ-Max	Del efficiency (100%-RQ)
PTG6	*MPK5*	19.70	0.12	74%	61%	89%	26%
PTG7	*MPK5*	20.51	0.06	77%	65%	92%	23%
PTG9	*MPK5*	19.70	0.05	89%	82%	97%	11%
CK	*MPK5*	19.61	0.18	100%			
PTG6	*UBI*	20.02	0.12				
PTG7	*UBI*	20.84	0.15				
PTG9	*UBI*	20.30	0.04				
CK	*UBI*	20.38	0.14				

The fragment deletion efficiency at the *MPK5* locus was estimated with qPCR using genomic DNA as a template and a pair of specific primers that encompass the gRNA2 cut site within *MPK5* (Fig. S1A). Because the *MPK5* locus with fragment deletion would not be amplified, the deletion (Del.) efficiency could be estimated as 100%-RQ. The same genomic DNAs in Fig. 3B were used and the *UBI* gene serves as the reference for relative quantification. Ct, threshold cycle; SD, standard deviation; RQ, relative quantity; RQ-Min and RQ-Max indicate the 95% confidential interval of RQ.